

# Feline Herpesvirus Pneumonia: Investigations Into the Pathogenesis

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Josep Maria Monne Rodriguez<sup>1</sup>, Gail Leeming<sup>1</sup>, Kernt Köhler<sup>2</sup>,  
and Anja Kipar<sup>3,4</sup>

## Abstract

Feline herpesvirus type 1 (FeHV-1) is one of the etiological agents of feline respiratory disease. FeHV-1 is an epitheliotropic and cytopathic virus that mainly causes rhinitis and conjunctivitis, although pneumonia is also occasionally seen. In this study, the authors investigated the pathogenesis of FeHV-1-associated pneumonia, comparing natural cases with viral infection of tracheal ring and cell cultures in vitro, using histology, immunohistology, double immunofluorescence, and transmission electron microscopy as investigative tools. The results confirm that FeHV-1 targets both respiratory epithelial cells and pneumocytes and indicate that FeHV-1 pneumonia is the consequence of continuous cell-to-cell viral spread from the upper airways via the trachea into the lungs. They provide strong evidence that FeHV-1-infected cells die primarily via apoptosis, following loss of cell-to-cell contact, rounding, and detachment. However, virus-induced lesions in vivo are dominated by marked neutrophil infiltration and extensive necrosis with less prominent apoptosis; in the airways, the tissue necrosis can extend into the submucosa. The necrosis appears to result from virus-induced neutrophil influx and release of proteolytic enzymes, such as matrix metalloproteinase-9, from the neutrophils.

## Keywords

apoptosis, cell-to-cell spread, feline herpesvirus 1, feline viral rhinotracheitis, pathogenesis, necrosis, neutrophil influx, pneumonia, respiratory diseases, cats

Feline herpesvirus 1 (*Felid herpesvirus 1*; FeHV-1) is an alpha-herpesvirus of the genus *Varicellovirus*, with double-stranded DNA and a glycoprotein-lipid envelope.<sup>41</sup> FeHV-1 was first isolated in 1958 and all further FeHV isolates described since have similar antigenic features, thus constituting one serotype.<sup>7,13,44</sup> FeHV-1 infects members of the family Felidae; although the domestic cat is the main host, cheetahs, lions, and pumas can also become infected.<sup>13</sup> Alongside feline calicivirus (FCV), FeHV-1 is the main pathogen of upper respiratory tract disease in which rhinitis and conjunctivitis are the main clinical features, known as cat flu. In the majority of cases, the disease is caused by either or both viruses; however, other etiological agents such as *Bordetella bronchiseptica* and *Chlamydomphila felis* are also frequently involved.<sup>12</sup>

FeHV-1 is excreted in nasal or ocular secretions of infected cats and is naturally transmitted via the oral, nasal, or conjunctival route, resulting in markedly higher FeHV-1 prevalence in multi-cat establishments.<sup>1,3,9,12,18</sup> Clinical presentation and severity depend on the viral strain as well as the age and immune status of the individual animal, accounting for the generally higher susceptibility of younger animals to infection.<sup>1,3,12,18,52,53</sup> Frequent clinical signs are sneezing and oculonasal serous discharge, which may be accompanied by hypersalivation with drooling, depression, inappetence, and pyrexia.<sup>12</sup> The discharges gradually become mucopurulent, and in severe cases, dyspnea may be

accompanied by coughing.<sup>12</sup> Clinical signs usually resolve within 10 to 20 days; however, severely affected animals can develop secondary bacterial infections that lead to chronic suppurative rhinitis and conjunctivitis.<sup>12,43,52</sup> In animals that recover from clinical disease, the virus can become latent in the trigeminal ganglia, rendering the cats prone to spontaneous episodes of upper respiratory disease due to viral reactivation.<sup>12</sup> Occasionally, particularly in young, debilitated cats, fatal primary FeHV-1-induced fibrinonecrotising bronchopneumonia can occur.<sup>5</sup>

The histological lesions caused by FeHV-1 have been described in both naturally and experimentally infected cats

<sup>1</sup>Division of Pathology, School of Veterinary Science, University of Liverpool, Liverpool, UK

<sup>2</sup>Institute of Veterinary Pathology, Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Giessen, Germany

<sup>3</sup>Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

<sup>4</sup>Institute of Global Health, University of Liverpool, Liverpool, UK

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## Corresponding Author:

Anja Kipar, Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 268, 8057 Zürich, Switzerland.  
Email: [anja.kipar@uzh.ch](mailto:anja.kipar@uzh.ch)

**Table 1.** Antibodies, Antigen Retrieval, and Detection Methods for Immunohistology (A) and Double Immunofluorescence (B).

A. Primary Antibodies, Antigen Retrieval, and Detection Methods for Immunohistology		
Antibody (clone)	Antigen Retrieval	Detection Method
Mouse anti-FeHV (FHV 7-7) <sup>a</sup>	None	HRP (Envision <sup>b</sup> )
Mouse anti-human MMP-2 (A-Gel VC2) <sup>c</sup>	CB pH 6.0, 96°C, 30 min	HRP (Envision <sup>b</sup> )
Mouse anti-human MMP-9 (IIA5) <sup>c</sup>	CB pH 6.0, 96°C, 30 min	PAP (Mouse <sup>d</sup> )
Mouse anti-human $\beta$ -catenin ( $\beta$ -Catenin-1) <sup>b</sup>	CB pH 9.0, 97°C, 20 min	HRP (Envision <sup>b</sup> )
Rabbit anticleaved caspase-3 (Asp175) <sup>e</sup>	CB pH 6.0, 96°C, 30 min	PAP (Rabbit <sup>f</sup> )
B. Primary Antibodies, Antigen Retrieval, and Fluorescence-Labeled Antibodies for Double Immunofluorescence		
Antibody (clone)	Antigen Retrieval	Fluorescence Antibody, Incubation Conditions
Mouse anti-FeHV (FHV 7-7). <sup>a</sup> 60 min, RT	CB pH 6.0, 98°C, 20 min	Alexa Fluor-488 (goat anti-mouse). <sup>g</sup> 30 min, RT
Rabbit anticleaved caspase-3 (Asp175). <sup>e</sup> 60 min, RT	None	Alexa Fluor-546 (goat anti-rabbit). <sup>g</sup> 30 min, RT

Abbreviations: CB, citrate buffer; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; PAP, peroxidase antiperoxidase; RT, room temperature.

<sup>a</sup>Custom Monoclonals International, Sacramento, CA, USA.

<sup>b</sup>Dako, Glostrup, Denmark.

<sup>c</sup>Thermo Fisher Scientific, Astmoor, Runcorn, United Kingdom.

<sup>d</sup>Jackson ImmunoResearch, West Grove, PA, USA.

<sup>e</sup>Cell Signaling Technology, Leiden, The Netherlands.

<sup>f</sup>Covance, Leeds, United Kingdom.

<sup>g</sup>Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA.

and include necrosis of the respiratory and alveolar epithelium, which occasionally extends into the submucosa and can induce lysis of the turbinate bones.<sup>4,5,8,10,11,19,20,37,50</sup> In both the upper and lower respiratory tracts, infection is often accompanied by abundant fibrin exudation and suppurative inflammation.<sup>8,19,21,23</sup>

Alphaherpesviruses ( $\alpha$ -HV), such as herpes simplex virus type 1 (HHV-1), varicella-zoster virus (HHV-3), and pseudorabies virus (SuHV-1), have been shown to spread from cell to cell by releasing new virions at the cell junction, which promotes the infection of adjacent cells. Accordingly, they show a tropism for polarized cells with extensive cell-to-cell contact, such as epithelial cells.<sup>27</sup> So far, information on the mode of FeHV-1 spread is limited, but in vitro studies have also provided evidence of cell-to-cell spread for this virus.<sup>35</sup>

Several in vitro tissue culture models have been proposed as alternatives to experimental infection of cats to study FeHV-1 pathogenesis, including tracheal organ cultures, tracheal mucosal explants, and conjunctival and corneal explants.<sup>35,36,42</sup> FeHV-1 replicates in vitro in both respiratory and conjunctival epithelial cells, but inconsistently in corneal explants.<sup>36,42</sup> The aim of the present work was to compare naturally infected cats with FeHV-1-induced pneumonia and suitable in vitro models of FeHV-1 infection (tracheal ring cultures and cell cultures) to examine the mode of viral spread from the upper to the lower respiratory tract and the mechanism of cell death (apoptosis and/or necrosis). The comparison of the lesions seen in FeHV-1 pneumonia with those in FeHV-1-infected tracheal organ cultures was employed to test the hypothesis that the host immunological response significantly contributes to the lesions observed in vivo; a better understanding of how and why a viral infection that is most commonly associated with

relatively benign upper respiratory tract disease can progress to fatal pneumonia will assist the prevention and treatment of this infection in susceptible individuals.

## Material and Methods

### Animals and Tissues

The study was performed on 21 predominantly young cats that had died or were euthanized due to clinical signs of (upper) respiratory disease and had been submitted for a diagnostic postmortem examination. Animals were of various breeds and ranged from 10 days old to adult, with an approximately equal sex distribution. Information on breed, sex, and age, where available, is shown in Supplemental Table 1. A full necropsy was performed and samples from all major organs as well as macroscopically altered tissues were collected, fixed in 10% nonbuffered formalin for 24 to 72 hours, trimmed, and routinely embedded in paraffin wax for histological and immunohistological examinations. In all cases, FeHV-1 pneumonia was diagnosed based on the demonstration of viral antigen within lung lesions by immunohistology (IH); in 11 cases, FeHV-1 was also cultured from the lungs, and in 4 cases, virions were demonstrated in the lung tissue by transmission electron microscopy (TEM; negative staining technique) (Supplemental Table 1). A bacteriological examination of the lungs was performed in 13 cases. Bacteria from the normal commensal population were isolated in 7 cases; in 5 cases, species likely to represent postmortem bacterial growth were cultured; and in the last case, no bacteria were isolated.<sup>34</sup> One lung tested negative for *Chlamydia sp* by polymerase chain reaction (Supplemental Table 1).

## In Vitro Studies

Tracheal organ cultures from 6 cats that had been euthanized for reasons other than respiratory disease were prepared and cultured in a submerged, rotary system as previously described in detail.<sup>35</sup> After 24 hours in culture, the tracheal cultures that had maintained complete and consistent cilia movement were infected with FeHV-1 (strain B927) at a multiplicity of infection of  $\approx 1$ , an inoculating dose that ensured continued viral replication for the entire examination period of up to 72 hours, as assessed by both virus isolation and real-time polymerase chain reaction.<sup>14,35</sup> Mock-infected rings from the same trachea were maintained under the same conditions and served as controls.

At the end of the culture period, tracheal rings were collected and either fixed in 4% paraformaldehyde (PFA) for 24 hours and routinely embedded in paraffin wax for histological and immunohistological examination or fixed in 4% PFA with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and routinely embedded in epoxy resin for TEM.

Feline embryo A (FEA) cells infected with a FeHV-1 strain isolated by Porter et al<sup>43</sup> were maintained for 24 hours in culture, washed with phosphate buffered saline, and centrifuged to form a cell pellet that was subsequently fixed in 4% PFA for 48 hours and routinely embedded in paraffin wax.<sup>26</sup>

## Histology, Immunohistology, and Immunofluorescence

Consecutive 3- to 5- $\mu$ m sections were prepared from the paraffin-embedded tissue specimens and stained with hematoxylin and eosin or used for IH and immunofluorescence to demonstrate FeHV-1 antigen, cleaved caspase-3 (as a marker of apoptosis), matrix metalloproteinase (MMP)-2 and -9, and  $\beta$ -catenin. For IH, the peroxidase antiperoxidase and the horseradish peroxidase methods were performed as previously described.<sup>6,31–33</sup> Briefly, after deparaffinization and rehydration, antigen retrieval, and blocking of endogenous peroxidase, sections were incubated with the primary antibodies at 4°C overnight and the secondary antibodies (30 minutes at room temperature) followed by the specified detection method used to visualize reactions with 3,3'-diaminobenzidine and counterstained with hematoxylin as detailed in Table 1A. Consecutive sections, incubated with nonreactive monoclonal or polyclonal antibodies, as well as the uninfected tracheal rings stained for FeHV-1 antigen served as negative controls. The following were used as positive control tissues: FeHV-1-infected FEA cell pellets (for FeHV-1), feline mammary carcinoma (for MMP-2 and -9), a feline lymph node (for cleaved caspase-3), and an unaltered feline lung (for  $\beta$ -catenin).

For double immunofluorescence, deparaffinized sections from the FeHV-1-infected FEA cell pellet underwent incubation with anti-FeHV-1 followed by anticlaved caspase-3 antibody, with appropriate antigen retrieval and fluorescence-labeled secondary antibodies (Table 1B). Nuclei were stained with 4',6-diamidino-2'-phenyl-indole dihydrochloride.

## Results

### FeHV-1 Pneumonia Results From the Extension of Upper Respiratory Tract Lesions Along the Airways

Similar to previously published descriptions, FeHV-1-induced pneumonia was characterized by necrotizing to necrosuppurative bronchitis, bronchiolitis, and alveolitis with occasional intranuclear viral inclusion bodies, neutrophil-dominated leukocyte infiltration, and variable fibrin exudation.<sup>5</sup> The affected cats also exhibited rhinitis and tracheitis of a similar nature (Figs. 1–6). Closer examination confirmed that lung lesions were a continuum of those present in the upper respiratory tract. They represented individual focal lesions that typically contained an affected bronchus and bronchiole from which necrosis and inflammation extended into the surrounding alveoli, with frequent evidence of a direct continuation (Figs. 3, 4).

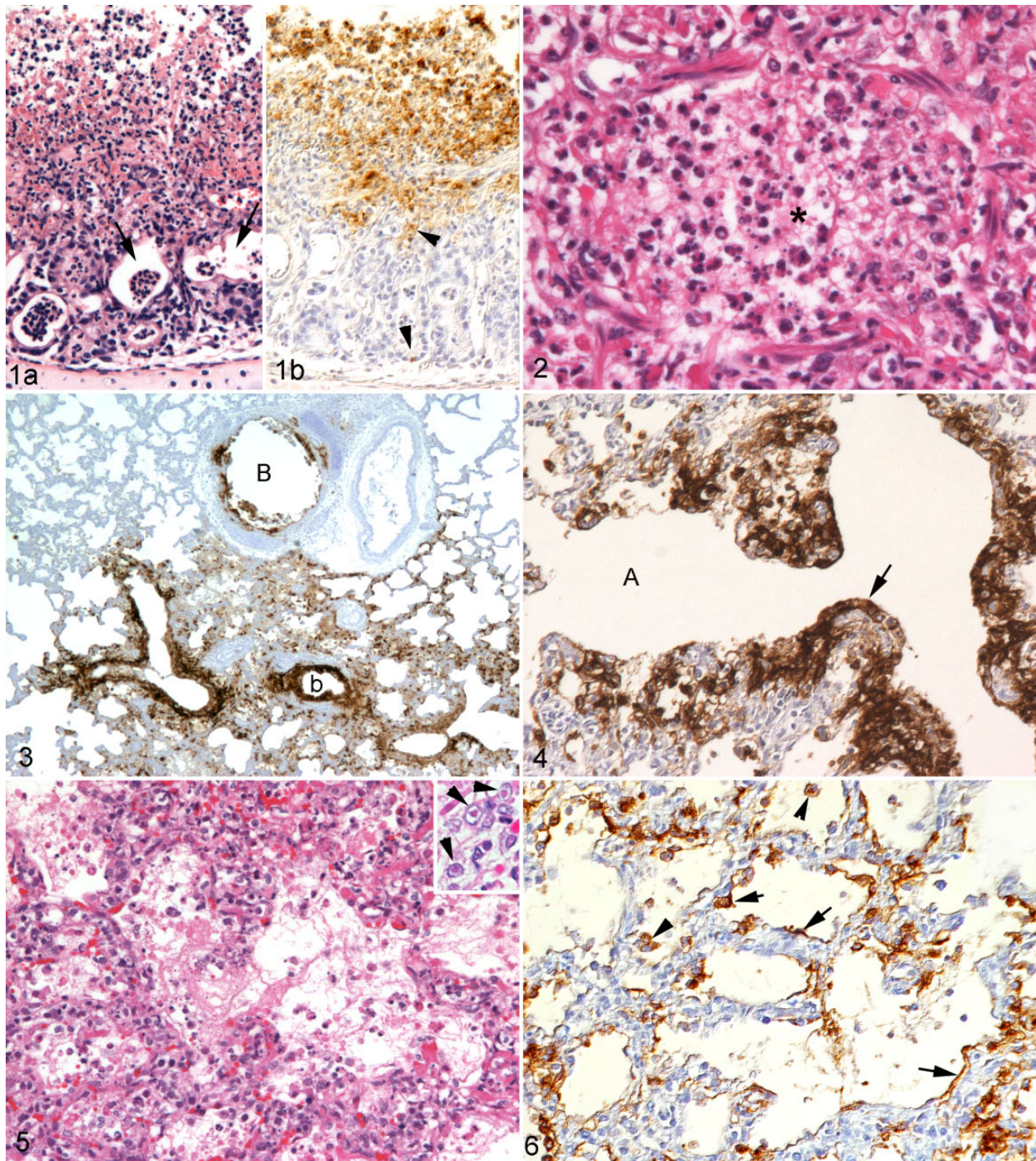
Immunohistology revealed FeHV-1 antigen in the respiratory epithelial cells of the airways, type I and II pneumocytes, and epithelial cells of the bronchial glands. Viral antigen was primarily seen within the cytoplasm but sometimes also in the nucleus of infected cells (Figs. 1b, 3, 4, 6).

Infected epithelial cells showed a range of morphological features, from unaltered cells within mildly affected areas to degenerating cells characterized by cytoplasmic swelling and detached cells with morphological features of apoptosis or necrosis (Fig. 2). In addition, viral antigen was occasionally found in alveolar macrophages within alveoli where degenerate infected epithelial cells and free viral antigen were also often present, suggesting that these macrophages had phagocytosed extracellular virions released from dead pneumocytes (Fig. 6).

### FeHV-1 Induces Apoptosis of Infected Cells and Spreads From Cell to Cell

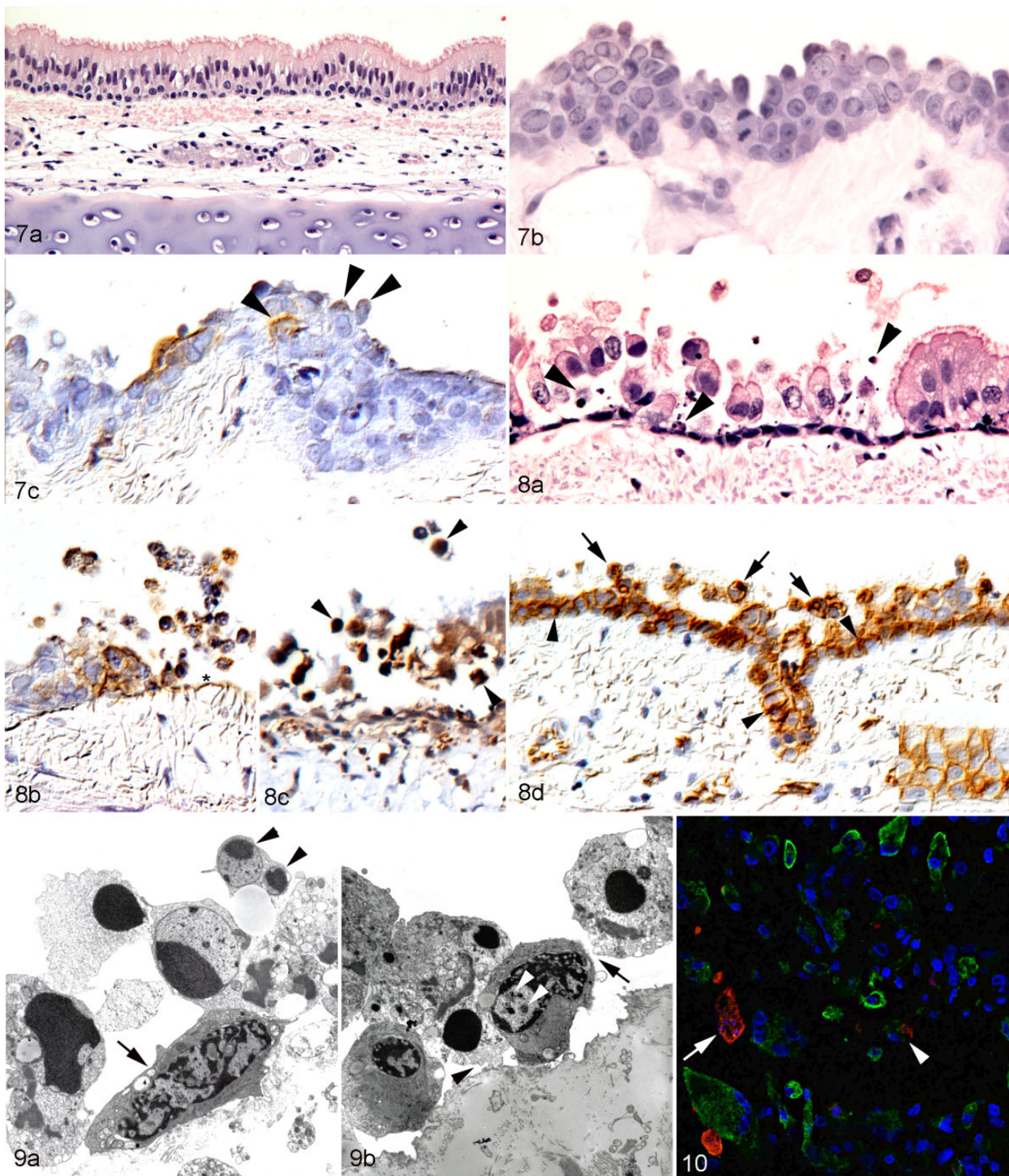
Natural pneumonia cases showed a plethora of changes, which sometimes included a significant inflammatory response. Therefore, to assess the direct viral cytopathic effect and exclude any potential effects of an inflammatory response, FeHV-1-infected tracheal ring cultures were examined at defined time points (24, 48, and 72 hours postinfection [hpi]). These suggested a sequence of cytopathic processes. At 24 hpi, the respiratory epithelium showed multiple foci of infection characterized by loss of cilia and rounding up of epithelial cells that had lost their pseudostratified columnar arrangement (Fig. 7a–c). Viral intranuclear inclusion bodies were occasionally seen. At the later time points, larger foci of infection with rounding of cells that appeared to have detached and areas with cell loss were observed. In some cases, a layer of uninfected basal cells remained (Fig. 8a). Detached cells contained viral antigen and often showed shrinkage, blebbing, and fragmentation, with chromatin margination and fragmentation (apoptosis) and expression of the apoptosis marker cleaved caspase-3 (Fig. 8b, c).<sup>55</sup> These changes were associated with an increase in  $\beta$ -catenin expression along the lateral aspects of the plasma





**Figures 1—6.** FeHV-1 pneumonia, cat, lung. **Figure 1.** Necrotizing bronchitis, bronchus, case No. 9. (a) The necrotic respiratory epithelium is replaced by a marked neutrophilic inflammatory infiltrate that extends to the bronchial glands, which also exhibit loss of epithelial cells (arrows). Hematoxylin and eosin (HE). (b) FeHV-1 antigen is detected in the necrotic respiratory epithelial cells and cell free in the mucosa and in glandular epithelial cells (arrowhead). Immunohistochemistry (IHC) for FeHV-1 antigen. **Figure 2.** Necrotizing bronchiolitis, case No. 19. The respiratory epithelium is effaced and replaced by necrotic debris, fibrin, and neutrophils. Asterisk: bronchiolar lumen. HE. **Figure 3.** Focal necrotizing pneumonia, case No. 4. FeHV-1 infection extends from the bronchus (B) to an associated bronchiole (b) and continues into the alveoli. IHC for FeHV-1 antigen. **Figure 4.** FeHV-1 spread across the bronchiolo-alveolar transition, case No. 16. Infection stretches from the respiratory epithelial cells (arrow) into the alveoli (A). IHC for FeHV-1 antigen. **Figure 5.** Necrotizing alveolitis, case No. 18. Alveoli contain necrotic cells and a large amount of fibrin. Inset: Focus with intranuclear inclusion body formation in epithelial cells (arrowheads). HE. **Figure 6.** Necrotizing alveolitis, case No. 15. The virus infects both type I (long arrows) and type II (short arrow) pneumocytes and possibly also alveolar macrophages (arrowheads). IHC for FeHV-1 antigen.





**Figures 7—10.** Cultured tracheal rings. **Figure 7.** 24 hours postinfection (hpi). (a) Mock-infected tracheal ring. The respiratory epithelium exhibits an intact cilia lining. Hematoxylin and eosin (HE). (b, c) FeHV-1-infected tracheal ring. Focus of infection with rounding up of epithelial cells and loss of pseudostratified columnar arrangement. (b) HE. (c) Viral antigen is detected within the cytoplasm of individual rounded epithelial cells (arrowheads). Immunohistochemistry (IHC) for FeHV-1 antigen. **Figure 8.** 72 hpi. Large focus of infection. (a) The majority of epithelial cells are detached from the neighboring cells and the basal cell layer. Cells are rounded up and some show morphological features of apoptosis (arrowheads). HE. (b) FeHV-1 infects all respiratory epithelial cells; there is a complete loss of epithelium with exposure of the submucosal surface (asterisk). IHC for FeHV-1 antigen. (c) Rounded and detached epithelial cells express the apoptosis marker cleaved caspase-3 (arrowheads). IHC for cleaved caspase-3. (d) The expression of  $\beta$ -catenin increases along the lateral aspect of the plasma membrane (arrowheads) and

membrane and, in completely detached cells,  $\beta$ -catenin translocation from the membrane to the cytoplasm (Fig. 8d). Ultrastructural changes included an increase in the nuclear:cytoplasmic ratio, loss of cilia, loss of cytoplasmic organelles, increased electron density of the cytoplasm, nuclear condensation, and apoptotic bodies (Fig. 9a). Detached cells were devoid of desmosomes and hemidesmosomes (Fig. 9b). Viral particles were present in the nuclei and very rarely within the cytoplasm of detaching cells (Fig. 9b).

To confirm that FeHV-1 induced apoptosis of infected cells, double immunofluorescence was applied to FeHV-1-infected cultured FEA cell pellets. At 24 hpi, a few infected cells were found to express both viral antigen and cleaved caspase-3, that is, to undergo apoptosis (Fig. 10); some of these cells also exhibited typical morphological features of apoptosis (shrunken cells with condensed nuclei).

Similar features were seen in naturally occurring cases of pneumonia; FeHV-1-infected respiratory epithelium exhibited  $\beta$ -catenin translocation and underwent apoptosis (Fig. 11a–d). In both the pneumonia cases and tracheal ring cultures, there was abrupt transition between infected and noninfected, and between degenerate and morphologically unaltered, epithelial cells (Fig. 11b, inset). Furthermore, in tracheal organ cultures, the increased size of the infected foci at 72 hpi indicated that cell loss appeared to expand circumferentially. In FeHV pneumonia, there was evidence of similar cell-to-cell spread of viral infection from the airways to adjacent alveoli, where infected cells were in close contact with each other, and also from the respiratory epithelium to the underlying bronchial glands (Fig. 1).

### *FeHV-1–Induced Lesions Are Associated With a Neutrophil-Dominated Inflammatory Response and Can Extend Beyond the Epithelium*

The FeHV-induced cytopathic changes in pneumonia cases were generally accompanied by a variably intense acute inflammatory response, ranging from abundant fibrin exudation with only a few inflammatory cells to a densely cellular infiltration (Figs. 1a, 2, 5, 12, 13a). The inflammatory infiltrate was predominantly neutrophilic, with a variable number of foamy macrophages as well as occasional lymphocytes and plasma cells. Neutrophils infiltrated the airway respiratory epithelium and submucosa, including the glandular structures, and were present in abundance within alveolar lumina (Figs. 1a, 2, 12). Neutrophils were most prominent in areas with severe tissue destruction and less frequent in older lesions, which exhibited marked thickening of

the alveolar septa, type II pneumocyte hyperplasia, and a predominantly mononuclear infiltrate.

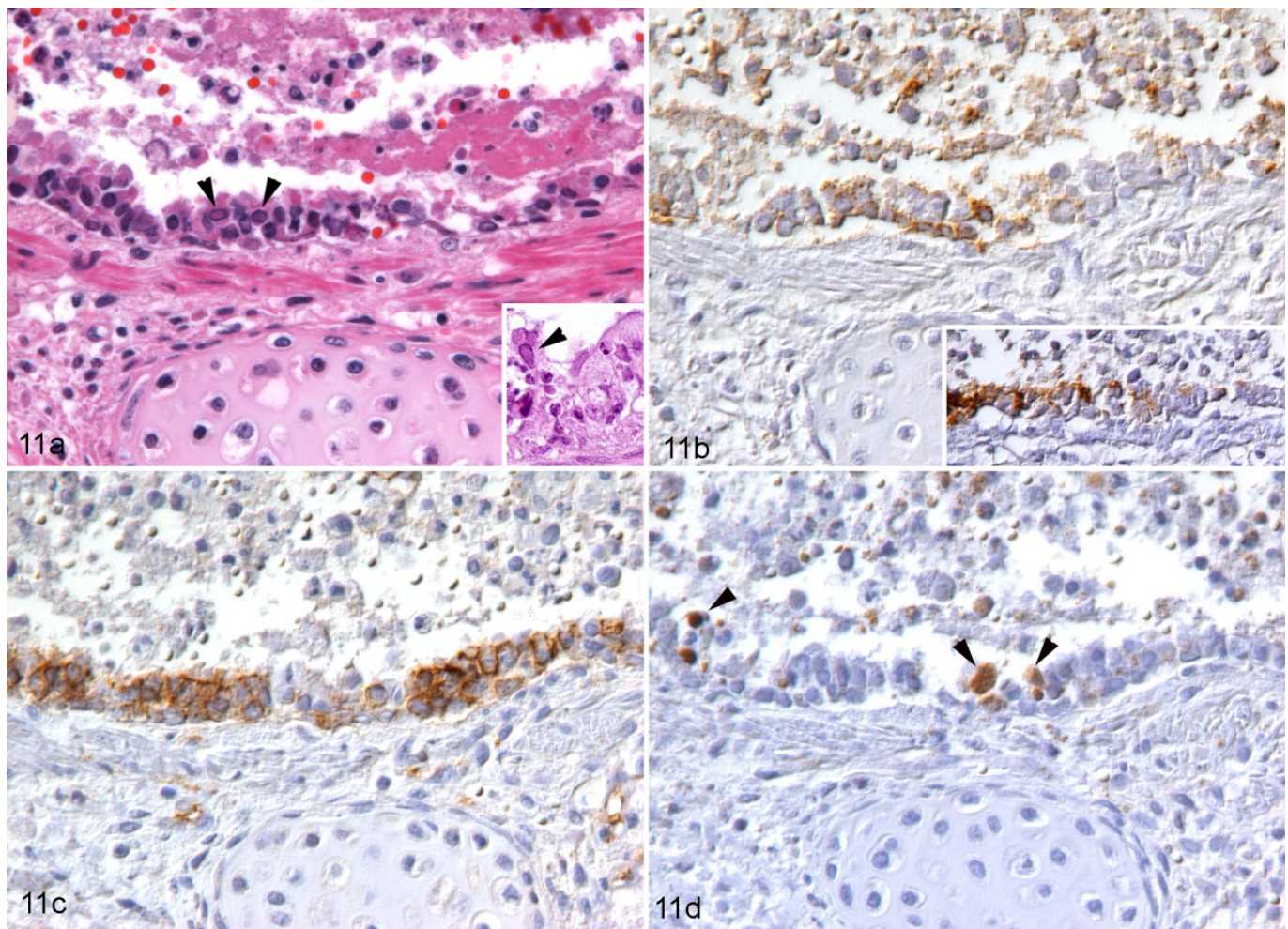
Inflammatory processes were accompanied by activation of endothelial cells in pulmonary vessels, indicated by their plump appearance and intense MHC class II expression (data not shown). Numerous band neutrophils and occasional monocytes were often seen adhering to the endothelial surface, indicating cell recruitment into the tissue. Frequently, marked perivascular edema was seen within and around the lesions.

Within inflammatory lesions, numerous epithelial cells, most of which were infected, showed morphological features of necrosis (cellular swelling, disruption of the plasma membrane, pyknosis, and karyorrhexis) and apoptotic cells were rare (Figs. 2, 12). In several cases, necrosis of the airways extended beyond the respiratory epithelium into the submucosa, occasionally associated with thrombosis or necrosis of submucosal blood vessels (Fig. 13). Four cases (case Nos. 4, 15, 18, 21) exhibited excessive necrosis that completely effaced all preexisting structures and extended to the surface of the bronchial cartilaginous rings (Fig. 13a). This was accompanied by an intense suppurative inflammation and the accumulation of abundant extracellular viral antigen admixed with cell debris above the cartilage surface (Fig. 13b). In contrast, cell death in FeHV-1-infected tracheal rings never extended beyond the respiratory epithelium (Fig. 8a–c).

Expression of the gelatinases MMP-2 and -9 was investigated as a potential mechanism of the extensive tissue destruction. In both infected and control tracheal rings, respiratory epithelial cells, fibrocytes, and chondrocytes exhibited variably intense MMP-2 expression. Lymphocytes and plasma cells, present in low numbers both in vessels and in the submucosa, were negative or weakly positive. In severely damaged areas, detached epithelial cells, submucosal fibroblasts, and, to a lesser extent, chondrocytes exhibited mild MMP-2 upregulation. MMP-9 expression was minimal in both uninfected and infected tracheal rings with no significant variation after FeHV-1 infection. In contrast, pneumonia cases exhibited strong MMP-9 expression in neutrophils and macrophages and mild to moderate expression by respiratory epithelial cells, pneumocytes, and chondrocytes (Figs. 14, 15). MMP-2 was less intensely expressed, but epithelial cells, submucosal fibrocytes and chondrocytes in the airways, and fibrocytes and epithelial cells, mainly in areas of severe necrosis and inflammation, exhibited a moderate reaction (Figs. 16, 17). Within areas of complete tissue destruction, staining for both MMPs was seen in the necrotic debris.

**Figure 8 (continued).** accumulates in the cytoplasm of rounded and detaching epithelial cells (arrows). Inset: Uninfected tracheal ring with ciliated pseudostratified columnar epithelial cells that exhibit  $\beta$ -catenin expression along the lateral aspect of the epithelial cells. IHC for  $\beta$ -catenin. **Figure 9.** 48 hpi. Ultrastructural features. (a) Epithelial cells show loss of cell contact, are rounded up, and show features of apoptosis, like nuclear condensation. Apoptotic bodies (arrowheads) are also seen. There is also an unaltered basal cell (arrow). Transmission electron microscopy (TEM). (b) Rounding and detaching epithelial cells lose contact with adjacent cells, indicating loss of desmosomes (arrow), and are detached from the base, indicating loss of hemidesmosomes (arrowhead) and features of apoptosis. A basal cell exhibits intranuclear viral particles (white arrowheads). TEM. **Figure 10.** Feline embryo A cell culture infected with FeHV-1, 24 hpi. Among infected, FeHV-1 antigen-positive (green) cells are some that undergo apoptosis; there is an example of a cell in the early stage of apoptosis, with only focal cleaved caspase-3 (orange-red) expression (arrowhead) and a cell in late apoptosis, with fragmented nucleus (arrow). Double immunofluorescence on formalin-fixed, paraffin-embedded cell pellet, with 4',6-diamidino-2'-phenyl-indole dihydrochloride (blue) nuclear stain.





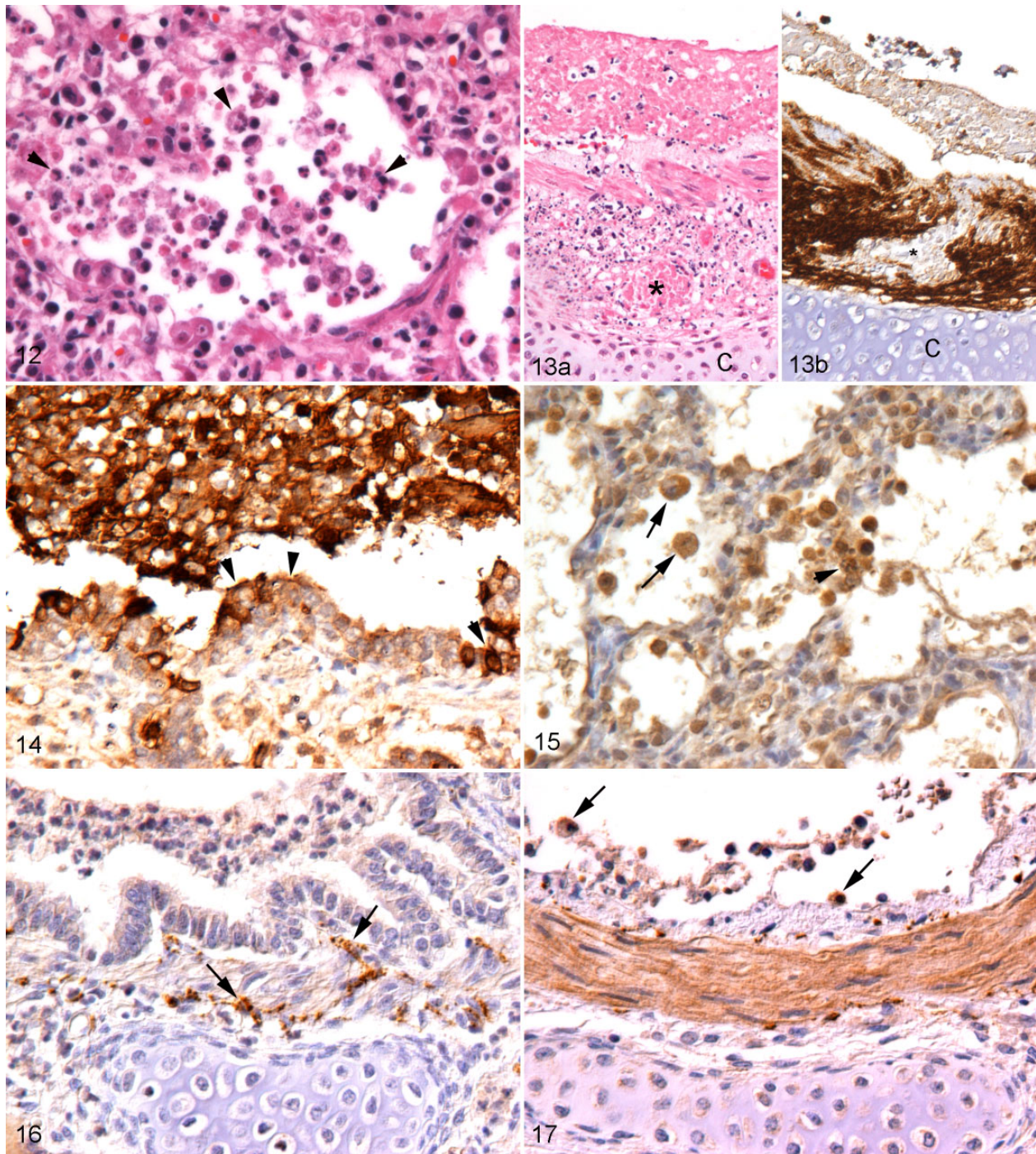
**Figure 11.** FeHV-1 pneumonia, cat, lung. Necrotizing bronchitis, case No. 1. (a) Epithelial cells appear shrunken and hypereosinophilic and often slough into the lumen, where they are admixed with fibrin and few inflammatory cells. Some epithelial cells exhibit intranuclear inclusion bodies (arrowheads, also in inset). Hematoxylin and eosin. (b) Numerous respiratory epithelial cells, aligned and detached, are found to be FeHV-1 infected. Inset: In another bronchiole, abrupt transition is seen between FeHV-1 infected and noninfected epithelial cells. Immunohistochemistry (IHC) for FeHV-1 antigen. (c) Epithelial cells in the affected area exhibit increased  $\beta$ -catenin expression along the lateral aspect of the plasma membrane or  $\beta$ -catenin accumulation in the cytoplasm. IHC for  $\beta$ -catenin. (d) Several rounded and shrunken epithelial cells in the affected area are confirmed to undergo apoptosis, based on the expression of cleaved caspase-3 (arrowheads). IHC for cleaved caspase-3.

## Discussion

Feline herpesvirus 1 is an important cause of upper respiratory disease in cats and occasionally also causes pneumonia, but always in association with upper respiratory tract lesions (rhinitis and tracheitis).<sup>5,11,37,45,50</sup> This was demonstrated in older experimental studies where upper respiratory disease was followed by lung involvement.<sup>8,23,29</sup> Our results are in accordance with these findings and suggest that FeHV-1 pneumonia is the consequence of viral spread along the respiratory tract. There was strong evidence that FeHV-1 first infects lower airways and then spreads to adjacent alveolar pneumocytes, resulting in a distinct multifocal, airway-centered lesion pattern that differs from that seen with naturally occurring FCV pneumonia and the pneumonia that develops after experimental FCV infections using aerosols or in response to other viruses that reach the lungs with the blood, such as cowpox virus,

together representing the various potential routes of viral pulmonary infection.<sup>22,40,49</sup> These findings indicate that, like other  $\alpha$ -HV, FeHV-1 spreads from cell to cell.<sup>27</sup> Members of the  $\alpha$ -HV family commonly show a tropism for polarized cells with extensive cell-to-cell contact, which allows the release of virions at the lateral cell junctions and subsequent infection of the adjacent cells.<sup>27</sup> In accordance with previous studies, our in vitro results from FeHV-1 infection of tracheal ring cultures provide further evidence that FeHV-1 behaves in a similar manner.<sup>35,36</sup> At 24 hpi, we observed several foci of infection with cell degeneration and death, which appeared to expand circumferentially with time; the virus was always found in morphologically intact cells at the border with the uninfected epithelium. This also suggests that the virus is released prior to cell degeneration and death, a feature described for another  $\alpha$ -HV, Marek's disease virus (GaHV-2).<sup>46</sup>





**Figures 12—17.** FeHV-1 pneumonia, cat, lung. **Figure 12.** Necrotizing and suppurative alveolitis, alveolus, case No. 1. The alveolar lumen contains degenerate epithelial cells and abundant neutrophils (arrowheads). Hematoxylin and eosin (HE). **Figure 13.** Necrotizing bronchitis, bronchus, case No. 15. (a) The necrosis extends from the respiratory epithelium into the submucosa, where it involves the blood vessels (asterisk) and reaches the surface of the bronchial cartilage (C). HE. (b) FeHV-1 antigen is seen within degenerating cells and cell free within the necrotic tissue, accumulating on the surface of the bronchial cartilage (C). Asterisk: lumen of necrotic vessel. Immunohistochemistry (IHC) for FeHV-1 antigen. **Figure 14.** Necrosuppurative bronchitis, bronchus, case No. 8. Inflammatory cells in the bronchial lumen and infiltrating the respiratory epithelium exhibit strong matrix metalloproteinase (MMP)-9 expression. The respiratory epithelium is also positive for MMP-9 (arrowheads). IHC for MMP-9. **Figure 15.** Necrosuppurative alveolitis, alveolus, case No. 15. Alveolar lumina contain several alveolar macrophages (arrows) and variable numbers of neutrophils (arrowhead); these show intense MMP-9 expression. Pneumocytes also appear MMP-9 positive. IHC for MMP-9. **Figure 16.** Unaltered bronchus, case No. 8. In an area with unaltered respiratory epithelium, submucosal fibrocytes show mild to moderate MMP-2 expression (arrows). IHC for MMP-2. **Figure 17.** Necrotizing bronchitis, bronchus, case No. 18. Submucosal fibrocytes beneath the necrotic epithelium exhibit intense MMP2 expression; some sloughed epithelial cells are also positive (arrows). IHC for MMP-2.



The present study confirms the epitheliotropism of FeHV-1. In the tracheal rings, only respiratory epithelial cells and sub-mucosal tracheal glands became infected. In some cases, there was evidence that viral replication and production were most intense in the upper layers, suggesting that FeHV-1, like other  $\alpha$ -HV, has a preference for well-differentiated and polarized epithelial cells.<sup>27</sup> In naturally infected cats, the virus was found in respiratory and glandular epithelial cells of the airways and alveolar type I and II pneumocytes. Viral antigen was mainly seen in the cytoplasm and only occasionally in the nucleus of infected cells, corresponding to the replication site of  $\alpha$ -HV (nucleus) and subsequent transport of the progeny virus to the lateral domains of the cells.<sup>27,48</sup> FeHV-1 antigen was also found in alveolar macrophages in areas of extensive virus-associated necrosis. Other  $\alpha$ -HV such as bovine herpesvirus 1 (BoHV-1) and SuHV-1 have been shown to infect macrophages; however, we and others observed macrophages in naturally infected animals admixed with cellular debris and cell free virus, which suggests that FeHV-1 antigen in the alveolar macrophages is the result of phagocytosis.<sup>2,5,25</sup>

The in vitro part of the present study illustrates that FeHV-1-infected cells undergo apoptosis. Using cultured tracheal rings, we could show a sequence of morphological changes: after loss of cilia, (hemi)desmosome-mediated polarization, and cell contact, cells detached from neighboring cells and basement membrane and sloughed off. This was accompanied by ultrastructural changes consistent with apoptosis. In FeHV-1-infected FEA cells (the cell line commonly used for FeHV-1 isolation), double immunofluorescence for cleaved caspase-3 and virus antigen confirmed apoptosis as the form of direct virus-induced cell death.<sup>26</sup> Comparable changes have been described in other in vitro models of  $\alpha$ -HV infection, that is, HHV-1 induced similar rounding up and detachment of infected epithelial cells in human nasal mucosal transplants; however, these changes were not clearly associated with apoptosis.<sup>15</sup> We also found apoptosis in the natural FeHV-1 pneumonia cases, where sloughed respiratory epithelial and alveolar cells with morphological evidence of apoptosis and cleaved caspase-3 expression were present.

We used immunohistological detection of  $\beta$ -catenin to further characterize the FeHV-1-induced cytopathic changes.  $\beta$ -catenin is a multitask protein involved in both maintenance of the cell structure (ie, epithelial polarization) and cell cycle signaling and is expressed on the cytoplasmic aspect of the cell membrane.<sup>38</sup> In vitro and in vivo mouse models have demonstrated that  $\beta$ -catenin regulates the epithelial repair after lung injury, showing an increase of signaling in epithelial cells during the transmigration of neutrophils across the alveolar epithelium.<sup>56</sup> It has also been suggested that  $\beta$ -catenin overexpression can induce apoptosis, rendering it part of a physiological mechanism to eliminate cells from the population.<sup>30</sup> We sometimes observed an increase in  $\beta$ -catenin expression at the lateral borders of epithelial cells that had started to round up and an accumulation of the protein in the cytoplasm of completely detached cells. Under physiological conditions,  $\beta$ -catenin is constantly phosphorylated and kept at a low concentration in

order to stabilize the cell-to-cell junction. Following cell damage, phosphorylation of  $\beta$ -catenin ceases and the protein accumulates in the cytoplasm and then translocates to the nucleus, where it promotes cell division.<sup>39</sup> In the current study, the overexpression of  $\beta$ -catenin was associated with cell damage, suggesting that this may represent part of the apoptotic pathway in infected cells.

All FeHV-1 pneumonia cases exhibited substantial neutrophil-dominated inflammation. This was seen without evidence of secondary bacterial infection. Similarly, an initial neutrophilic response was previously described in experimentally infected germ-free cats, suggesting that neutrophil recruitment is a direct consequence of the viral infection.<sup>23</sup> This is supported by a study using BoHV-1, where infected epithelial cells secreted IL-1 and TNF- $\alpha$ , which induced the transmigration of neutrophils across the bronchial epithelial cell layer.<sup>47</sup> Furthermore, upregulation of acute-phase inflammatory cytokines, including neutrophil chemoattractants, has been reported in cats with FeHV-1-induced rhinitis.<sup>28</sup> In our in vitro infected tracheal cultures, we saw infection of the respiratory epithelium, but the virus never spread beyond the basement membrane. Equid herpesvirus 1 (EHV-1) shows a similar behavior, whereas HHV-1, SuHV-1, and BoHV-1 have been shown to cross the basement membrane.<sup>15,16,51,54</sup> For FeHV-1, the behavior seems to vary depending on the in vitro system, since viral spread across the basement membrane was reported in conjunctival and tracheal explants using the FeHV-1 strain C27, grown in Crandell Rees feline kidney cells for an unknown number of passages prior to inoculation.<sup>36</sup> More recent, authors have attributed these variations in behavior to the virus strain and the temperature at which the tissue was cultured.<sup>42</sup> Furthermore, the tracheal organ cultures in the present study were complete tissue rings, as opposed to the small explants used by Li et al,<sup>36</sup> which has been suggested to be a factor in the differences in infection in corneal explants observed in different models.<sup>42</sup> For SuHV-1, destruction of the basement membrane was associated with the presence of a trypsin-like serine protease; however, whether the enzyme was of cellular or viral origin was not clear.<sup>17</sup> In our study, there was clear evidence of a direct association between viral infection, the neutrophil-dominated inflammation, and tissue necrosis, suggesting that cellular enzymes, such as MMP-2 and -9, were involved in the processes.<sup>39</sup> It is interesting that we did not see significant MMP-9 and MMP-2 expression in the tracheal in vitro model, although there was evidence of mild upregulation in the infected epithelium. In contrast, there was consistent strong MMP-9 expression in FeHV pneumonia, predominantly in neutrophils and macrophages. In human periodontal disease caused by HHV-1, it has been demonstrated that opsonization of the virus leads to neutrophil recruitment, which in turn amplifies the tissue destruction by increasing the upregulation of MMP-9.<sup>24</sup>

In FeHV-1 pneumonia, the necrotizing lesions were seen in association with a neutrophilic inflammatory response. The necrosis was often extensive and partly extended into the underlying tissues, reaching the bronchial cartilage in severe

cases. This differs substantially from the FeHV-1 lesions seen in the in vitro tracheal cultures, where they were mainly centered on the epithelium and characterized by apoptosis. The neutrophilic response in the natural cases might play a role in the development of necrotizing lesions. This is indicated by the increase of MMP-9 expression. What induces the suppurative inflammation is still not completely understood; infected intact and necrotic epithelial cells, alveolar macrophages, and the virus itself could all participate by releasing acute inflammatory cytokines. Likewise, the mechanism of cell destruction needs to be further understood as other intracellular substances (ie, lysosomal enzymes) released from neutrophils and macrophages are probably involved. The differences between the natural in vivo cases and in vitro models highlight the advantages of a model in which the systemic immune response is absent (to allow the study of direct viral effect) and the limitations, as this response can be a significant influence in the progression and outcome of the viral infection.

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